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Metabolic Control Analysis and engineering of the yeast sterol biosynthetic pathway

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Introduction

Sterols are essential components of eukaryotic membranes and contribute to important physiological characteristics, such as osmotic robustness and hormonal effects on growth (Daum *et al.*, 1999), but most importantly, the sterol pathway has shown to be a highly effective target for antifungal drug development. The incidence of life-threatening fungal infections has been increased, particularly among patients who are immuno-compromised by Human Immunodeficiency Virus infection (HIV causing AIDS) and those receiving immuno-suppressive therapy for organ transplantation or chemotherapy for cancer. Resistance among various fungal pathogens in these patients to antifungal drugs has been a matter of concern. Therefore, development of more effective antifungal agents has become a priority.

Our research focuses on the yeast sterol biosynthesis. The type of sterol observed varies between the Kingdoms of Life; in yeast it is ergosterol. Starting from 2-aceto-CoA, ergosterol is synthesized by a sequence of at least 21 enzymes, among them two different cytochrome-P450 heme oxygenases. One P450, lanosterol C-14α demethylase (sterol biosynthetic step 13), encoded by the yeast ERG11-gene, is specifically inhibited by the antifugal compound fluconazole. This particular step (13) is suggested to be important for fungal growth. However, how control on ergosterol biosynthesis in yeast is distributed among this and all other steps in the pathway is still unknown. In the current report, we present the first results of a systematic approach to quantify metabolic control of the ergosterol biosynthesis in the yeast *Saccharomyces cerevisiae*.

Method

Mutant system and high-throughput analysis

Through modulation of the ERG11 expression in a constructed mutant around the level of a reference strain, we were able to determine the effect of this pathway enzyme on the specific growth rate and sterol concentrations. The *ERG11* gene has been put under control of a regulatable promoter, for this purpose a doxycycline-repressible *tetO*$_7$*-CYC1* promoter was used (a kind gift from Professor Hegemann). This promoter is down-regulated in the presence of doxycycline and expresses transcript in the absence of doxycycline at levels comparable to genes controlled by the strong GAL1 promoter (Gari,1997). Thus this mutant allows ideal study of high- and low-level expression of the gene relative to that of the wild type (reference strain YUG37). The effect of doxycycline on the maximum specific growth rate (μ_{max}) of the mutant was determined in aerobic batch cultures by a Bioscreen C instrument; a high-throughput system in which 200 separate cultures each have a working volume of just 100 μl (Bioscreen: http: // www. All microbiology . com/index.shtml).

Results

When cultivated without any doxycycline, the μ_{max} of the *ERG11*-overexpressing mutant was more than 15% higher than the reference yeast strain YUG37 (μ_{max} 0.37 and 0.32 h^{-1}, respectively; see Figure 1A). Upon supplying the culture medium with different amounts of doxycycline, the mutant showed a decrease in the rate of biomass synthesis, i.e., its specific growth rate was impaired (see Figure 1B). The reference strain YUG37 did not show any toxic effects on growth over the concentration-range of doxycycline used (data not shown).

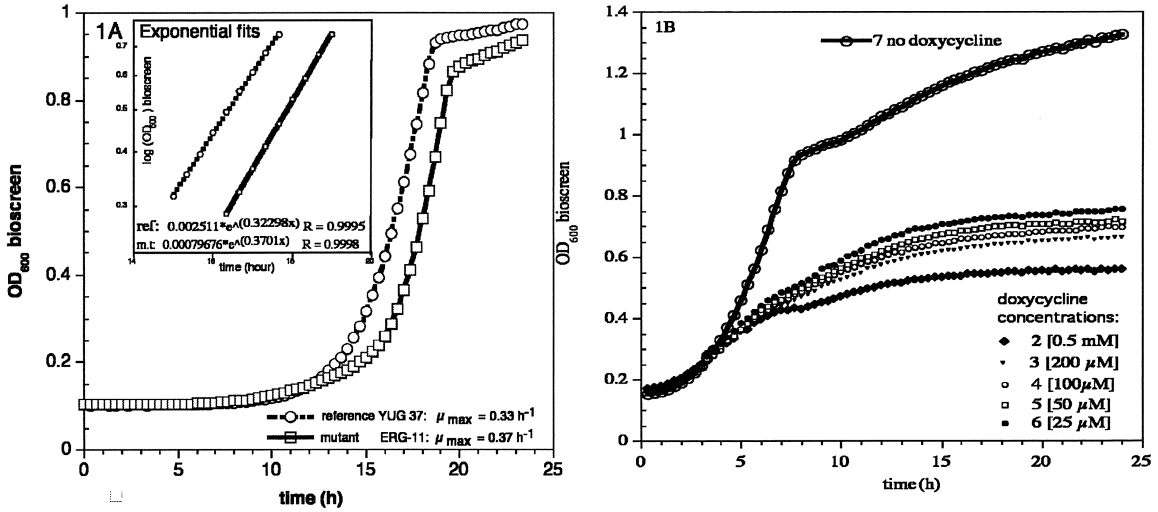

Figure 1A. Biomass (measured as OD_{600}) as a function of time (hour) of reference strain YUG 37 (▬○▬) and mutant strain ERG-11 (▬□▬). *Both strains were cultivated on complex medium (YEPD 2% glucose) as an aerobic batch in a high-throughput bioscreen system. The maximum specific growth rate (μ_{max}) was estimated 0.32 h^{-1} and 0.37 h^{-1} for YUG37 and ERG11, respectively (i.e, an average value estimated during exponential growth-phase for 8 different cultures of each strain). The inset shows one of the exponential fits for each strain; 1B. Biomass of mutant strain ERG11 as a function of time. Mutant strain ERG11 was cultivated on the medium with different amounts of doxycycline (range 0-500 μM as indicated in the figure). The increase of biomass was impaired by doxycycline for mutant ERG-11, whereas no such effect was found for reference strain YUG37 (data shown in figure 2B: experiment number 42).*

In the absence of doxycycline and in the presence of a strong *tetO$_7$-CYC* promoter (comparable with a GAL1 promoter), a higher level of ERG11 gene-product was expected in the mutant strain. As a consequence, this mutant (ERG-11) should be less sensitive to the antifungal agent fluconazole (which specifically inhibits the ERG11 gene-product and causes growth inhibition) than the reference strain (YUG-37). To test the difference in expression level of *ERG11*, we cultivated both strains on medium containing fixed amounts of fluconazole without any doxycycline. Indeed the mutant was less sensitive to different amounts of fluconazole than the referent strain (see figure 2A). This supported our hypothesis of a higher *ERG11* expression level in the mutant strain.

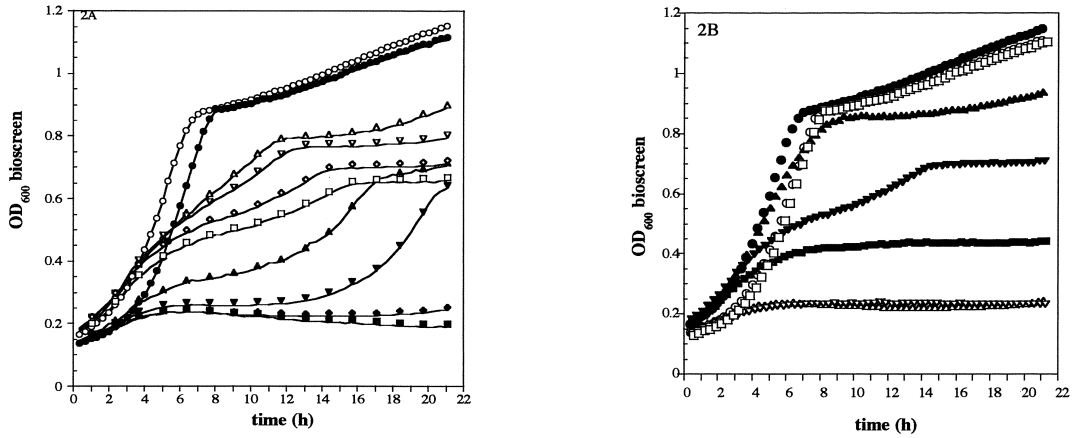

Figure 2A. Biomass (measured as OD_{600}) of mutant (ERG-11) and reference strain (YUG37) as a function of time (in hour) cultivated on complex medium (without any doxycycline) supplemented with different amounts of fluconazole (range 0-250 μM: —○—22: ERG11 w/o fluconazole (F); —●—2: YUG37 w/o F; —△—95: ERG11+[F]=50μM; —▲—75: YUG37+[F]=50μM; —▽—94: ERG11+[F]=100μM; —▼—74: YUG37+[F]=100μM; —◇—92: ERG11+[F]=200μM; —◆—72: YUG37+[F]=200μM; —□—91: ERG11+[F]=250μM; —■—71: YUG37+[F]=250μM. At each fluconazole concentration the mutant showed better growth results, indicating a higher *ERG11* expression level.. **2B. Biomass of mutant ERG11 and reference strain YUG37 as a function of time**. Both strains were cultivated on complex medium supplemented with a fixed amount of fluconazole (fluconazole=[F]=200μM) and with different amounts of doxycycline (concentration 0 and 200 μM were used: —●— 22: ERG11 w/o any of both inhibitors —○— 42: YUG37 w/o F+Doxycycline=[D]=200μM; —▲—62 ERG11 w/o F+[D]=200μM; —△—72: YUG37+[F]=200μM w/o D; —▼— 92ERG11+[F]=200μM w/o D; —▽—102: YUG37+both [F]=[D]=200μM; —■—122 ERG11+both [F]=[D]=200μM; —□—2 YUG37 w/o any of both inhibitors. In the mutant strain the sensitivity to fluconazole increased when more doxycycline was added to the medium. The numbers refer to the different wells, i.e., 100 μl culture vessels used in the bioscreen.

To test the doxycycline-repression of *ERG11* in the ERG11-mutant, we supplied the batch cultures of both strains with or without one fixed amount of fluconazole together with or without 200 μM of doxycycline. As expected, as doxycycline was added, the more growth was sensitive to fluconazole in the mutant strain (see figure 2B).

These early results indicate that the levels of cytochrome-P450 heme oxygenase (Lanosterol C-14α demethylase, sterol-biosynthetic step 13) in the *ERG11* mutant strain could indeed be modulated by doxycycline and that it appears to have significant control on the specific growth rate of *S. cerevisiae*.

Quantification of protein and metabolite levels are underway to determine flux- and concentration-control coefficients. Together with computer simulation of the kinetics of this biochemical network in *Gepasi* (Mendes & Kell 1998), we will determine the distribution of control and regulation within the sterol biosynthetic pathway in yeast.

References

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